nature neuroscience

Cerebellar Purkinje cell activity drives motor learning

T D Barbara Nguyen-Vu[1,2,7], Rhea R Kimpo[1,7], Jacob M Rinaldi[1], Arunima Kohli[1], Hongkui Zeng[3], Karl Deisseroth[4–6] & Jennifer L Raymond[1]

The climbing fiber input to the cerebellar cortex is thought to provide instructive signals that drive the induction of motor skill learning. We found that optogenetic activation of Purkinje cells, the sole output neurons of the cerebellar cortex, can also drive motor learning in mice. This dual control over the induction of learning by climbing fibers and Purkinje cells can expand the learning capacity of motor circuits.

The climbing fiber input to the cerebellum from the inferior olive is widely viewed as the source of the instructive signals controlling the induction of cerebellum-dependent learning[1]. *In vitro*, climbing fiber activation can induce plasticity at synapses in the cerebellar cortex, and, *in vivo*, climbing fibers encode errors during a wide range of motor learning tasks[1]. Notably, electrical stimulation of the inferior olive *in vivo* can replace the unconditioned stimulus in a classical conditioning procedure, providing causal evidence for a role of climbing fibers in the induction of learned changes in behavior[2]. However, such causal studies have not been replicated for motor skill learning, and recent findings have challenged the view that instructive signals encoded by the climbing fibers are the driver of cerebellum-dependent motor learning[3,4].

A second, candidate neural instructive signal in the cerebellum is the activity of Purkinje cells[5]. Purkinje cells encode information that could, in principle, be used to guide the induction of plasticity[6,7], and several models have suggested a role for Purkinje cell activity in guiding the acquisition or consolidation of motor memory[5,8,9]. However, causal evidence that Purkinje cell activity can induce learning *in vivo* has been lacking; previous attempts to induce learning by activating Purkinje cells with electrical stimulation of the cerebellar cortex were unsuccessful[10,11]. Thus, we harnessed the cell-type specificity of optogenetics and the power of the well-characterized vestibulo-ocular reflex (VOR) to determine whether Purkinje cell activity can drive the induction of learned changes in behavior.

The VOR is a reflexive eye movement response to a head movement (vestibular stimulus) that helps to stabilize images on the retina. If a vestibular stimulus is repeatedly paired with the movement of a visual stimulus, motor learning can adaptively modify the amplitude of the VOR to reduce image motion on the retina. The direction of the visual stimulus motion relative to the vestibular stimulus determines whether an adaptive increase or decrease in the VOR is learned[12]. Purkinje cell activity in the cerebellar flocculus reflects both the vestibular stimulus and the direction of the visual stimulus (the eye movements made to track the visual stimulus); thus, these neurons carry the information required to control the direction of learning[6,13]. We tested whether learned changes in the VOR could be induced by pairing a vestibular stimulus with direct activation of Purkinje cells in the absence of any visual stimulus.

When VOR learning is induced by pairing a vestibular stimulus with a visual stimulus, the timing, or phase, of activity in Purkinje cells relative to the vestibular stimulus encodes the required direction of the learning[6,13]. We compared the effect of optogenetically stimulating floccular Purkinje cells (**Supplementary Figs. 1** and **2**) during the contraversive phase of a sinusoidal vestibular stimulus with the effect of stimulating during the ipsiversive phase (**Fig. 1a**).This pairing was done in complete darkness (that is, in the absence of any visual cues that could guide associative motor learning). As a control, the vestibular stimulus was delivered in the absence of Purkinje cell stimulation during the 30-min training period. This vestibular-alone training resulted in a significant habituation of the VOR ($P = 0.0006$, one sample t test; **Fig. 1b**), as described previously[14].

Activation of Purkinje cells during the contraversive phase of the vestibular stimulus to roughly mimic the response observed in the majority of Purkinje cells during normal VOR increase training[6,13] was sufficient to drive learning. During training, there was a gradual increase in the VOR response amplitude relative to the vestibular-alone control ($P = 0.0002$, Tukey; **Fig. 1b** and **Supplementary Fig. 3**), with the VOR response being larger at the end of training than the pre-training baseline ($P = 0.03$, one sample t test; **Fig. 1b**). We assessed this VOR learning in the absence of Purkinje cell stimulation by briefly interrupting the optogenetic stimulation to measure the eye movement response to the vestibular stimulus alone.

The timing of the Purkinje cell activity relative to the vestibular stimulus was critical to the induction of VOR learning ($P = 0.0043$, contraversive versus ipsiversive Purkinje cell stimulation, Tukey; **Fig. 1b**). Purkinje cell activation induced VOR learning when paired with the contraversive phase of the vestibular stimulus, but, when paired with the ipsiversive phase of the vestibular stimulus, it had no significant effect beyond that induced by training with the vestibular stimulus alone ($P = 0.42$, Tukey; **Fig. 1b** and **Supplementary Fig. 3**). Thus, the motor learning induced by direct optogenetic activation of Purkinje cells was associative, as it only occurred when Purkinje cell activation was paired with the appropriate phase of the vestibular stimulus.

In addition to inducing learning, optogenetic activation of Purkinje cells could elicit eye movements and have an immediate effect on eye movement performance during training (**Supplementary Figs. 4** and **5a–c**). However, the learning induced by Purkinje cell activation, which was measured after training in the absence of Purkinje cell stimulation, was not a secondary consequence of its effects on the eye movement

[1]Department of Neurobiology, Stanford School of Medicine, Stanford, California, USA. [2]Department of Molecular and Cellular Physiology, Stanford School of Medicine, Stanford, California, USA. [3]Allen Institute for Brain Science, Seattle, Washington, USA. [4]Department of Bioengineering, Stanford University, Stanford, California, USA. [5]Department of Psychiatry, Stanford School of Medicine, Stanford, California, USA. [6]Howard Hughes Medical Institute, Stanford University, Stanford, California, USA. [7]These authors contributed equally to this work. Correspondence should be addressed to J.L.R. (jenr@stanford.edu).

Received 8 August; accepted 16 October; published online 27 October 2013; doi:10.1038/nn.3576

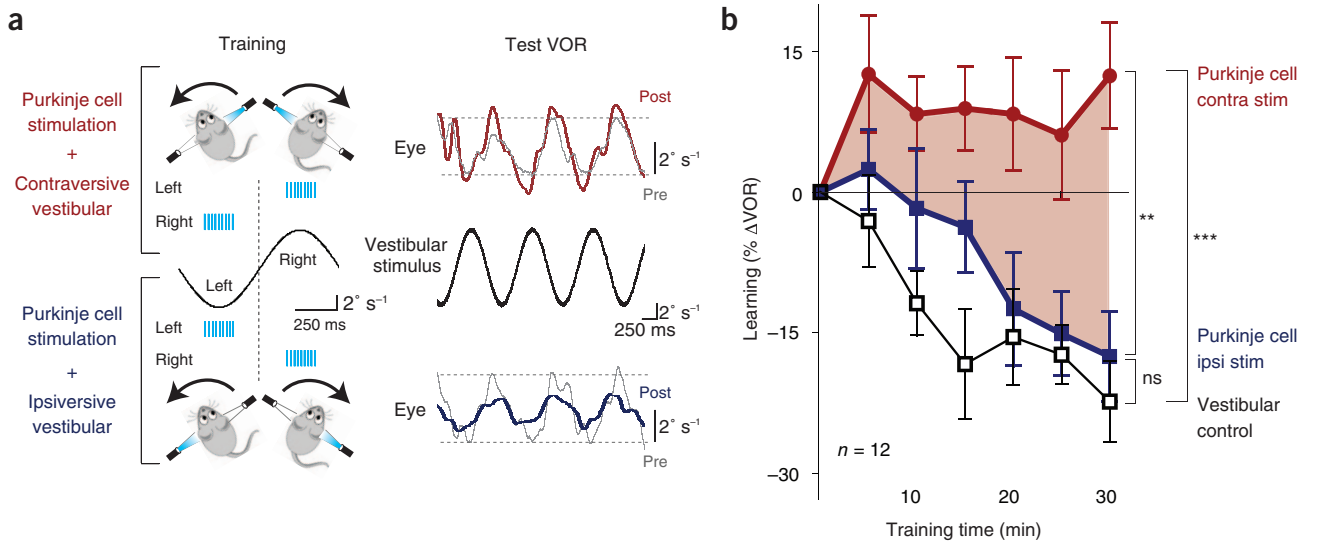

Figure 1 Purkinje cell activation induced associative motor learning. (**a**) Training procedures. A sinusoidal vestibular stimulus was used for training and testing (black trace, angular velocity of the head). During training, floccular Purkinje cells in the two hemispheres were stimulated (cyan) in an alternating fashion so that the activation of Purkinje cells in each hemisphere was paired with either the contraversive phase of the vestibular stimulus (red) or the ipsiversive phase of the vestibular stimulus (blue). The VOR was tested pre- and post-training by measuring the eye movement response to the vestibular stimulus in the absence of Purkinje cell stimulation. Example raw eye velocity traces illustrate results from one mouse that exhibited an increase in the VOR after training with contraversive Purkinje cell stimulation (red) and a decrease in the VOR after ipsiversive stimulation (blue) relative to pre-training (gray). (**b**) Motor learning, measured as the change in VOR amplitude post-training relative to pre-training, depended on the training condition ($F_{2,31} = 11.59$, $P = 0.0002$, two-way repeated measures ANOVA). Black open squares indicate that the vestibular stimulus was presented alone during training ($n = 10$). Red circles and blue squares indicate that Purkinje cell activation was paired with the contraversive or ipsiversive phase of the vestibular stimulus, respectively ($n = 12$). Data are presented as mean ± s.e.m. $^{**}P = 0.0043$, $^{***}P = 0.0002$, ns indicates not significant ($P = 0.42$).

performance during training, because there was no correlation between these two effects (**Supplementary Fig. 5**).

Our results provide causal evidence that Purkinje cell activation can contribute to the induction of motor learning, a role that has generally been ascribed to climbing fibers. Purkinje cell activity may induce plasticity in the cerebellar cortex[15,16], downstream in the vestibular nuclei[17–20] or both. In the Purkinje cell axons, the simple spikes elicited optogenetically should be indistinguishable from those elicited by parallel fiber input; however, in the Purkinje cell dendrites, optogenetic activation may cause more widespread calcium influx than would normally be triggered by parallel fiber input. Moreover, *in vitro*, direct depolarization of the Purkinje cells can, in some cases, substitute for climbing fiber activity, inducing calcium influx in the Purkinje cell dendrites and hence synaptic plasticity, particularly long-term depression at the parallel fiber–Purkinje cell synapses[15]. However, the VOR learning induced by Purkinje cell activation did not seem to result from plasticity mechanisms that would normally be triggered by climbing fiber activity, as climbing fiber activation and Purkinje cell activation did not substitute for each other. Purkinje cell activation paired with the contraversive phase of the vestibular stimulus induced a learned increase in the VOR (**Fig. 1b**). No such learned increase in the VOR was observed after training, when we paired optogenetic stimulation of the climbing fibers (**Supplementary Fig. 6**) with the same, contraversive phase of the vestibular stimulus (contraversive climbing fiber versus vestibular-alone control at 30 min, $P = 0.1052$, Mann-Whitney U; versus contraversive Purkinje cell stimulation, $P = 0.0278$, t test; **Figs. 1b** and **2**).

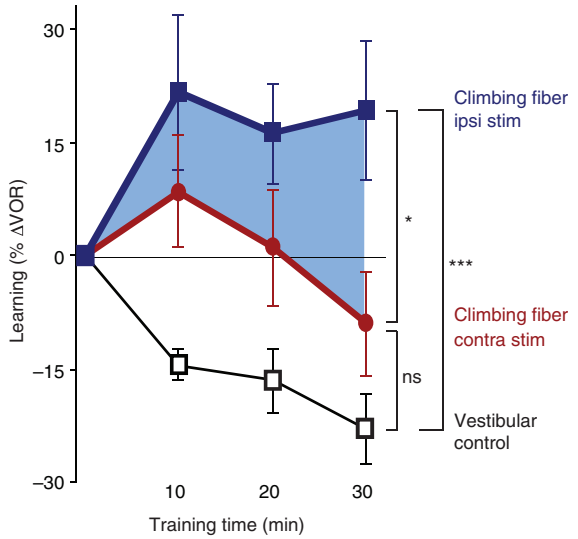

Figure 2 Climbing fiber activation induced motor learning with different timing than Purkinje cells. Motor learning induced by climbing fiber activation paired with the ipsiversive (blue squares, $n = 9$) or contraversive (red circles, $n = 8$) phase of the vestibular stimulus, compared with the control condition in which the vestibular stimulus was presented alone during training (black open squares, $n = 7$). Changes in the VOR relative to the pre-training baseline depended on the training condition ($F_{2,3} = 6.0$, $P = 0.0278$, Friedman test). Data are presented as mean ± s.e.m. $^{*}P = 0.0343$, $^{***}P = 0.0009$, ns indicates not significant ($P = 0.1052$).

Notably, climbing fiber activation induced an associative learned increase in the VOR when paired with the ipsiversive phase of the vestibular stimulus ($P = 0.0343$ versus contraversive climbing fiber stimulation, $P = 0.0009$ versus vestibular control, Mann-Whitney U; **Fig. 2**). Thus, error signals carried by the climbing fibers can have a causal role in motor skill learning, as previously demonstrated for classical conditioning[2]. However, climbing fiber stimulation was most effective at a time relative to the vestibular stimulus when Purkinje cell stimulation had no effect (ipsiversive stimulation; **Figs. 1b** and **2**), just as Purkinje cell stimulation was effective at a time when climbing fiber stimulation was relatively ineffective (contraversive stimulation; **Figs. 1b** and **2**). This double dissociation suggests that Purkinje cell activation and climbing fiber activation *in vivo* can induce learning through distinct mechanisms.

We found that motor memory for an increase in the VOR could be artificially implanted by stimulating either the climbing fibers or the Purkinje cells. Purkinje cells and climbing fibers encode different task-related signals during oculomotor learning[6,13], yet they can achieve a similar behavior outcome, namely, an increase in the amplitude of the VOR. Thus, joint control of the induction of motor skill learning by cerebellar Purkinje cells and climbing fibers may expand the capacity of the motor circuit to learn in response to different cues.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank E. Knudsen, T. Moore, C. Shatz, D. Madison, G. Zhao, O. Winter, S. Umamoto, A. Adamantidis, M. Carter, H. Nguyen and R. Hemmati for discussions and assistance. This study was supported by grants from the US National Institutes of Health (RO1 DC04154, RO1 NS072406 and P01 NS053862) and the James S. McDonnell Foundation to J.L.R., from the US National Science Foundation Graduate Research Fellowship Program and US National Institutes of Health (F31DC010547) to T.D.B.N.-V., from the US National Institutes of Health and Defense Advanced Research Projects Agency to K.D., from the US National Institutes of Health (K01 NS069617) to R.R.K., and from the US National Institutes of Health (P30 DC10363 and P30 NS069375) for imaging and virus core facilities.

AUTHOR CONTRIBUTIONS

T.D.B.N.-V. conducted all Purkinje cell experiments. R.R.K. conducted all climbing fiber experiments. J.M.R. conducted pilot experiments. A.K. helped with histology. K.D. and H.Z. provided reagents. J.L.R. supervised all aspects of the work.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

1. Ito, M. *Annu. Rev. Neurosci.* **12**, 85–102 (1989).
2. Mauk, M.D., Steinmetz, J.E. & Thompson, R.F. *Proc. Natl. Acad. Sci. USA* **83**, 5349–5353 (1986).
3. Ke, M.C., Guo, C.C. & Raymond, J.L. *Nat. Neurosci.* **12**, 1171–1179 (2009).
4. Catz, N., Dicke, P.W. & Thier, P. *Curr. Biol.* **15**, 2179–2189 (2005).
5. Miles, F.A. & Lisberger, S.G. *Annu. Rev. Neurosci.* **4**, 273–299 (1981).
6. Lisberger, S.G. & Fuchs, A.F. *Brain Res.* **69**, 347–353 (1974).
7. McCormick, D.A. & Thompson, R.F. *Science* **223**, 296–299 (1984).
8. Mauk, M.D. & Donegan, N.H. *Learn. Mem.* **4**, 130–158 (1997).
9. Shutoh, F., Ohki, M., Kitazawa, H., Itohara, S. & Nagao, S. *Neuroscience* **139**, 767–777 (2006).
10. Babalian, A.L. & Vidal, P.P. *J. Neurophysiol.* **84**, 2514–2528 (2000).
11. Heuer, H.W., Tokiyama, S. & Lisberger, S.G. *J. Neurophysiol.* **100**, 1320–1331 (2008).
12. Ito, M., Shiida, T., Yagi, N. & Yamamoto, M. *Brain Res.* **65**, 170–174 (1974).
13. Raymond, J.L. & Lisberger, S.G. *J. Neurosci.* **18**, 9112–9129 (1998).
14. Stahl, J.S. *J. Neurophysiol.* **91**, 2066–2078 (2004).
15. Crepel, F. & Jaillard, D. *J. Physiol. (Lond.)* **432**, 123–141 (1991).
16. Belmeguenai, A. *et al. J. Neurosci.* **30**, 13630–13643 (2010).
17. Pugh, J.R. & Raman, I.M. *Neuron* **51**, 113–123 (2006).
18. Menzies, J.R.W., Porrill, J., Dutia, M. & Dean, P. *PLoS ONE* **5**, e13182 (2010).
19. McElvain, L.E., Bagnall, M.W., Sakatos, A. & du Lac, S. *Neuron* **68**, 763–775 (2010).
20. Aizenman, C.D., Manis, P.B. & Linden, D.J. *Neuron* **21**, 827–835 (1998).

ONLINE METHODS

Mice. All experimental procedures were approved by the Administrative Panel on Laboratory Animal Care at Stanford University. Experiments were performed on male and female adult (≥8 weeks old) mice. All mice were housed on a reversed 12-h light/12-h dark cycle, and experiments were conducted during the mice's dark cycle. For the Purkinje cell activation experiments, L7-Cre mice[21] were obtained from Jackson Laboratory. The L7/pcp2 promoter drives selective expression in cerebellar Purkinje cells. To target ChR2 expression to cerebellar Purkinje cells, the L7-Cre mice were crossed with Ai32 mice[22] or injected in the cerebellar flocculi with virus (see below) carrying *loxP*-flanked ChR2. The Ai32 mice conditionally express an improved channelrhodopsin-2/EYFP fusion protein (ChR2 (H134R)-EYFP) from the endogenous Gt(ROSA)26Sor locus. Expression is enhanced by the presence of a CAG promoter. To target ChR2 expression to the floccular climbing fibers, an injection of viral vector (see below) carrying the ChR2 gene was targeted to the dorsal cap of Kooy, the subnucleus of the inferior olive that projects to the flocculi, of adult C57BL/6 male mice (≥9 weeks old, see below).

AAV virus injections. To drive ChR2 expression selectively in Purkinje cells, adeno-associated virus (AAV) carrying *loxP*-flanked ChR2-EYFP under the Ef1α promoter was injected into the cerebellum of L7-Cre mice[21]. The pAAV-EF1α *loxP*–flanked hChR2(H134R)-EYFP-WPRE-HGHpA[23] carries the *channelrhodopsin* (ChR2) gene fused to enhanced yellow fluorescent protein (ChR2-EYFP). ChR2-EYFP is in the reverse orientation between two nested pairs of incompatible lox sites, *loxP* and *lox2722*. Following introduction into the L7-Cre transgenics, Cre excises the *lox* sites and inverts the construct to allow transcription of ChR2-EYFP via the Ef1α promoter. The Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) enhances expression and the human growth hormone poly A (HGHpA) tail ensures translation. The recombinant AAV vectors were serotyped with AAV8 coat proteins and packaged by the viral vector core at the University of North Carolina.

pAAV-EF1α *loxP*–flanked hChR2(H134R)-EYFP-WPRE-HGHpA was injected into the cerebellar flocculi of L7-Cre mice to target ChR2 expression specifically to Purkinje cells of the cerebellar region responsible for VOR learning. Mice were anesthetized with ketamine/dexmedetomidine followed by isoflurane, and a craniotomy was made above the periotic capsule bilaterally to access the flocculi. 1.0 μl of the AAV solution was injected into each flocculus over the course of 10 min. Mice were allowed to recover for a minimum of 4 weeks to allow for expression before further surgeries or experimentation.

To express ChR2 in the relevant climbing fibers, we injected AAV-CaMKIIα-ChR2(H134R)-EYFP (Neuroscience Gene Vector and Viral Core, Stanford University; titer ≥ 10^{12}) into the inferior olive, stereotaxically targeting the dorsal cap of Kooy, which projects to the cerebellar flocculi. The CaMKIIα promoter drives expression in excitatory neurons. A 0.5–1.0-μl volume of viral particles was pressure-injected over 15–30 min. 6–8 weeks after the injection, mice were surgically prepared for behavioral experiments, and the climbing fibers were activated by illuminating the cerebellar flocculi.

Implant surgery for behavioral experiments. Mice were surgically prepared for behavioral experiments as previously described[24]. In brief, while under anesthesia with ketamine/dexmedetomidine followed by isoflurane, mice were implanted with a head post for restraining the head, an eye coil for measuring eye movements, and craniotomies were performed to access the cerebellar flocculi for optogenetic stimulation and recording. A custom-built head post was attached to the top of the skull using anchor screws and dental acrylic. A small, copper scleral search coil (IET), 1 mm in diameter, was implanted on the temporal side of one eye beneath the conjunctiva. The search-coil leads were threaded subcutaneously and soldered to a two-pin connector that was also cemented with dental acrylic. Craniotomies were made on the periotic capsule bilaterally, and cannulae (Plastic One) were implanted above the craniotomies using dental acrylic to allow access to the cerebellar flocculi. Mice were individually housed after surgery, and allowed to recover for 4–5 d before behavioral experiments.

VOR behavioral training and analysis. During each behavioral experiment, the head of the mouse was immobilized by attaching the implanted head post to a restrainer. The restrainer was attached to a turntable (Carco Electronics), which delivered a vestibular stimulus by rotating the mouse about an earth-vertical axis. Horizontal eye position was measured using the scleral search coil implanted in one eye. Measurements of eye movements were taken in 40-s blocks at a sampling rate of 500 or 1,000 Hz. Eye velocity was calculated by differentiating eye position measurements. The gain of the VOR was measured as the eye-movement response to a sinusoidal vestibular stimulus (1 Hz, ±10° s^{-1} peak velocity) in complete darkness (that is, in the absence of visual input). The amplitude and phase of the eye movement response were extracted from a sinusoidal fit to the eye velocity record, after excluding any segment containing a saccade or motion artifact. The VOR gain was calculated as the ratio of the eye-to-head movement amplitudes. Learning was calculated as the percent change in the gain of the VOR, measured in the dark, after each 5- or 10-min block of training.

Optical stimulation was delivered to each cerebellar flocculus via a 200-μm optical fiber, which was inserted through the implanted cannula and connected to a blue laser (473 nm, Laserglow). Based on published results[25], we estimate that the single fiber was sufficient to illuminate most of the ~1-mm^3 volume of the cerebellar flocculus. The optical fiber was sealed along its length to prevent light emission except for where the tip penetrated the brain. Placement of the optical fiber was guided by the use of light pulses; when possible, the fiber was placed at a site where the optogenetic stimulation evoked eye movements.

Separate cohorts of mice were used for the bilateral Purkinje cell stimulation and climbing fiber stimulation experiments. Each group of mice was tested once on each of the corresponding training conditions, with the order of experiments randomized across animals: 1) bilateral optogenetic Purkinje cell or climbing fiber stimulation during the contraversive phase of a vestibular stimulus; 2) bilateral optogenetic Purkinje cell or climbing fiber stimulation during the ipsiversive phase of a vestibular stimulus; 3) the vestibular stimulus alone. Contraversive and ipsiversive refer to head motion away from or toward the side of optogenetic stimulation, respectively. An additional cohort of mice underwent the same set of training procedures, but with unilateral rather than bilateral stimulation of Purkinje cells (**Supplementary Fig. 3**). For the Purkinje cell experiments, the bilateral training procedures were conducted using the L7-Cre transgenic mice crossed with the Ai32 ChR2 transgenic mice. The unilateral training procedures were conducted using the L7-Cre transgenic mice injected with AAV virus to express ChR2 in the Purkinje cells. Three of the mice in the unilateral stimulation cohort underwent some of the training procedures more than once, and the replications from an individual mouse were averaged and treated as a single data point. A subset of mice that underwent unilateral stimulation also received visual-vestibular training to increase or decrease the VOR (**Supplementary Fig. 7**). The visual-vestibular training procedures were interleaved with the optogenetic stimulation training procedures. Each mouse used in the climbing fiber stimulation experiments was tested once on each training condition, with the exception that health issues precluded the testing of one mouse on climbing fiber stimulation during the contraversive phase of the vestibular stimulus, and two mice on the vestibular alone control condition. There was a minimum of 2 d between training sessions. A subset of mice from the climbing fiber cohort underwent one (n = 2 mice) or two (n = 3 mice) behavioral experiments involving optogenetic climbing fiber stimulation before undergoing the behavioral experiments reported in this paper.

Behavioral training consisted of pairing a 1-Hz, ±10° s^{-1} sinusoidal vestibular stimulus in the dark with optogenetic stimulation during the contraversive phase of the vestibular stimulus (head movement away from the side of stimulation) or the ipsiversive phase of the vestibular stimulus (head movement toward the side of stimulation). For bilateral training, the flocculi in the two hemispheres of the cerebellum were optically stimulated in an alternating fashion so that the phase relationship (contraversive or ipsiversive) for stimulation on each side was maintained relative to the vestibular stimulus. For bilateral Purkinje cell stimulation training, the optical stimulation parameters were a 50-Hz train of 5-ms pulses for 420 ms with intensity ≤3 mW mm^{-2}, centered on peak contraversive or ipsiversive vestibular stimulus velocity and delivered on every cycle of the 1-Hz stimulus. These parameters were chosen to produce an increase in Purkinje cell firing rate roughly comparable to what occurs during visually and vestibularly driven eye movements (mice: mean firing rate ~25–60 Hz with peak-to-peak modulation of ~40–110 Hz[26–28]; other species: mean firing rate ~15–135 Hz with peak-to-peak modulation of ~75–240 Hz[13,29–31]). For unilateral Purkinje cell stimulation training, the optical stimulation parameters were similar, except we used a 66-Hz train of 10-ms pulses (four mice) or a 50-Hz train of 10-ms pulses (nine mice). For bilateral climbing fiber stimulation training, climbing fibers were activated

with a 250-ms train of three pulses of light (15-ms duration, 1–2 mW mm^{-2}) with a 125-ms inter-pulse-interval, centered on peak ipsiversive or contraversive vestibular stimulus velocity. At the end of each experiment with climbing fiber stimulation training, a train of high-frequency optical stimuli (20-Hz, 2-ms pulse duration) was delivered, to test for functional expression of ChR2 in climbing fibers and correct placement of the optical fiber. If no eye movement was elicited by high-frequency stimulation on at least one side, that experiment was excluded ($n = 4$ experiments). This criterion was established before data collection, based on pilot experiments not included in the present data set. Vestibular-alone control training consisted of a 1-Hz, ±10° s^{-1} sinusoidal vestibular stimulus in the dark with no optogenetic stimulation or visual stimulus. Investigators conducting the experiments were not blind to the training conditions.

Training to induce VOR learning was conducted in 5-min blocks (Purkinje cell stimulation) or 10-min blocks (climbing fiber stimulation). Between blocks, the VOR was tested by delivering the vestibular stimulus in the absence of optogenetic stimulation for 40 s.

Visual-vestibular training to decrease the VOR gain consisted of pairing a 1-Hz, ±10° s^{-1} sinusoidal vestibular stimulus with 1-Hz, ±10° s^{-1} sinusoidal rotation of an illuminated optokinetic drum (visual stimulus) in the same direction, such that the visual stimulus was stationary relative to the mouse and required a VOR gain of zero to stabilize the image on the retina. Training to increase the VOR gain consisted of pairing a 1-Hz, ±10° s^{-1} sinusoidal vestibular stimulus with oppositely directed 1-Hz, ±10° s^{-1} sinusoidal optokinetic drum rotation, such that a VOR gain of 2 would be required to stabilize the image on the retina.

To test the immediate effects of optogenetic Purkinje cell activation on eye movement performance during training, the eye movements made in response to the vestibular stimulus were measured in the presence and absence of the 50-Hz blue-light stimulus trains. The effect on performance was calculated as the percent change in eye movement amplitude observed immediately, when Purkinje cells were stimulated during the vestibular stimulus, as compared with the eye movement amplitude made in response to the vestibular stimulus alone before training. The discrete, optogenetically evoked eye movements shown in **Supplementary Figure 4** were elicited using a train of 5-ms, 20-Hz light pulses in the absence of vestibular stimulation to demonstrate the powerful effect of Purkinje cell activation to elicit as well as modify motor performance. In each mouse, the optogenetically evoked eye movement response was consistent across experimental days.

Statistical analysis. For the behavioral experiments, adequate sample size was determined from pilot experiments using unilateral stimulation of Purkinje cells and using unilateral stimulation of climbing fibers, based on the assumption (borne out by the results) that the effect size of bilateral stimulation would be at least as big as the effect of unilateral stimulation.

Data are presented as mean ± s.e.m. Statistical analyses were performed using Excel, Prism and Statview. All tests were two-sided. One-sample *t* tests were used to determine if a mean was significantly different from zero. Paired *t* tests were used to determine a significant change in mean across conditions or time. Unpaired Student's *t* tests were used to compare groups. Pearson's test was used to determine correlation.

The Lilliefors test (Matlab) was used to assess normality of the data. Based on this test, all data were judged to have been sampled from a normal distribution. Bartlett's test of homogeneity of variances was used to assess whether the variance was similar in groups being compared. The results of the Lilliefors and Bartlett's tests were used to select appropriate statistical analyses.

For the behavioral experiments testing the effects of training with Purkinje cell stimulation, Bartlett's test indicated similar variance across groups, therefore, a repeated-measures two-way ANOVA was performed, with time and training condition (Purkinje cell stimulation during the ipsiversive phase of the vestibular stimulus, Purkinje cell stimulation during the contraversive phase of the vestibular stimulus and vestibular-alone control) as factors. Separate ANOVAs were performed on the data sets from unilateral and bilateral Purkinje cell stimulation, which were performed in different cohorts of mice. In each case, a significant F ratio indicated the presence of significant differences between training conditions. We therefore performed *post hoc* tests to compare training conditions. The bilateral data had unequal sample sizes across training conditions; we therefore used the Tukey *post hoc* test (Prism). The unilateral data had equal sample sizes; we therefore used Fisher's LSD *post hoc* test (Prism).

For the behavioral experiments testing the effects of training with climbing fiber stimulation, Bartlett's test indicated that the variance of the results for the vestibular-alone control was not equal to the variance when the climbing fibers were stimulated, violating the equal variance assumption of the ANOVA. For this reason, we used the nonparametric Friedman test (Prism). A significant *P* value justified the use of *post hoc* Mann-Whitney U tests to compare training conditions (Statview). Because ANOVA is considered to be robust to unequal variances, we also conducted a repeated-measures two-way ANOVA on the climbing fiber data, which, similar to the Friedman test, indicated significant differences between training conditions (Statview).

***In vivo* optrode recording and analysis.** Extracellular electrophysiological recordings from Purkinje cells were made in awake mice during optogenetic stimulation using an optrode, which consisted of a tungsten electrode clamped to a 200-μm optical fiber identical to that used for behavioral training. Mice were placed in the behavioral apparatus as described above. An optrode was advanced through the implanted cannula to access the cerebellar flocculus. Purkinje cell activity was sampled at 50 kHz using Spike 2 (CED). Purkinje cells were identified on the basis of the electrophysiological waveform and relative position in the cerebellar laminar structure, and tested for their responsiveness to blue light stimulation.

For mice expressing ChR2 in Purkinje cells, trains of optical stimulation were used to test the effectiveness of the optogenetic stimulation to elevate Purkinje cell firing rate. We compared the average, spontaneous firing rate of each Purkinje cell during periods between trains of blue light stimulation with the average firing rate during the trains of the blue light stimulation. The trains of brief light pulses used in the behavioral experiments (50-Hz train of 5-ms pulses for 420 ms with intensity ≤ 3 mW mm^{-2}) achieved an increase in Purkinje cell firing rate comparable in amplitude and duration to what occurs during visual and vestibular oculomotor behaviors[26–32] (**Supplementary Fig. 2**).

In vivo extracellular recordings of Purkinje cells in the flocculus were also performed in mice expressing ChR2 in climbing fibers ($n = 7$ cells in 5 mice). Simple and complex spikes were sorted off-line (Spike 2). In some recordings, the optical stimulus created an electrophysiological artifact. In such cases, the waveform of the artifact was isolated either by delivering high-frequency light pulses (20 Hz) so that the complex spikes failed to follow every light pulse, or by moving the electrode (~200 μm) away from the cell and delivering the standard trains of three light pulses. The stimulus artifact was then subtracted from the electrophysiological records to obtain complex spike waveforms.

Histology. To visualize ChR2 expression in Purkinje cells or climbing fibers, mice were killed 4 or 6–8 weeks after virus injection, respectively. Mice were deeply anesthetized and immediately perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehye (wt/vol) in PBS. The brain was removed and post-fixed for 2 h at 20 °C. After fixation, the brain was placed in 30% sucrose (wt/vol) in PBS solution overnight at 4 °C. The brain was then embedded in OTC (Sakura Fine Tek) and frozen for cryosectioning. Coronal sections of 20 or 25 μm were made through the cerebellar flocculi, inferior olives or both. Purkinje cells and climbing fibers expressing ChR2-EYFP were imaged using a Nikon Eclipse E800 fluorescence microscope and identified by anatomical location and morphology.

ChR2-EYFP expression in Purkinje cells was close to 100% for the L7-Cre transgenic mice that were crossed to Ai32 ChR2 transgenic mice ($n = 3$ mice). ChR2-EYFP–expressing Purkinje cells were counted in the flocculi recovered from virus-injected L7-Cre mice that underwent behavioral experiments (range = 10–54%, mean = 26.3 ± 3.6%, $n = 11$ mice). Cells were counted from every other 20-μm section to avoid double-counting, and the percentage of cells expressing ChR2 was calculated as the number of positive Purkinje cells in each flocculus divided by a count of average total Purkinje cells in a flocculus from one representative mouse. Immunohistochemistry was used to counterstain for Purkinje cells to estimate the total number of Purkinje cells in the flocculi (estimate of 1,814 Purkinje cells per flocculus). Slices were incubated with blocking solution containing 10% normal goat serum (vol/vol) and 1% bovine serum albumin (wt/vol) in PBS with 0.3% Triton X-100 (PBS-T, wt/vol) for 1 h at 20 °C, and then with primary antibodies diluted in blocking solution overnight at 4 °C (polyclonal rabbit antibody to calbindin D-28K, AB1178, Millipore, A6455, 1:500). Slices were then washed three times with PBST and incubated with

doi:10.1038/nn.3576

secondary antibody (Alexa Fluor 594–conjugated goat antibody to rabbit, Invitrogen, A-11012, 1:1,000) for 1 h at 20 °C.

Brain sections from eight mice in the climbing fiber experiment were processed for YFP immunostaining as follows: sections were washed in PBS with 0.25% Triton X-100 (PBS-T) and blocked with 10% normal goat serum and 5% bovine serum albumin in PBS-T (wt/vol) overnight at 4 °C. Sections were incubated overnight at 4 °C in rabbit polyclonal antibody to GFP, which also binds to YFP (1:500 dilution, Invitrogen). Sections were then washed with PBS-T, incubated in a goat secondary antibody to rabbit conjugated to a fluorophore (1:250 dilution, Invitrogen) and washed with PBS-T. Immunostained sections were mounted with Prolong Gold, which also labeled cell nuclei with DAPI, a blue fluorescent stain (Invitrogen).

21. Barski, J.J., Dethleffsen, K. & Meyer, M. *Genesis* **28**, 93–98 (2000).
22. Madisen, L. *et al. Nat. Neurosci.* **15**, 793–802 (2012).
23. Sohal, V.S., Zhang, F., Yizhar, O. & Deisseroth, K. *Nature* **459**, 698–702 (2009).
24. Boyden, E.S. & Raymond, J.L. *Neuron* **39**, 1031–1042 (2003).
25. Aravanis, A.M. *et al. J. Neural Eng.* **4**, S143–S156 (2007).
26. Barmack, N.H. & Yakhnitsa, V. *J. Neurosci.* **31**, 9824–9835 (2011).
27. Yoshida, T., Funabiki, K. & Hirano, T. *Eur. J. Neurosci.* **25**, 1467–1474 (2007).
28. Hoebeek, F.E. *et al. Neuron* **45**, 953–965 (2005).
29. Lisberger, S.G. & Fuchs, A.F. *J. Neurophysiol.* **41**, 733–763 (1978).
30. Nagao, S. *Exp. Brain Res.* **77**, 531–540 (1989).
31. Pastor, A.M., De la Cruz, R.R. & Baker, R. *Prog. Brain Res.* **114**, 359–381 (1997).
32. Kahlon, M. & Lisberger, S.G. *J. Neurophysiol.* **84**, 2945–2960 (2000).